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Gabriel Nunez, M.D.  
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## INTRODUCTION

Cancer is the result of multiple genetic events, including activation of oncogenes and inactivation of tumor suppressor genes. The protein products of the former are often mitogens, whereas the products of the latter suppress proliferation. It is becoming increasingly apparent that tumor suppressor genes like p53 function in part by activating an apoptotic death pathway. In addition, certain oncogenes such as *bcl-2* appear to contribute to tumor development primarily by promoting abnormal cell survival via an apoptosis inhibitory signal. Thus, disruption of the apoptosis pathway appears integral to many malignancies including breast cancer. Furthermore, treatment of cancer with chemotherapy or radiation therapy is limited by the emergence of tumor cells resistant to these therapies. This resistance limits our ability to successfully treat these neoplasms.

The product of the *bcl-2* gene, which is activated via translocation in B-cell lymphomas, is an important modulator of apoptosis in many types cell lineages and tissues (1). *Bcl-2* encodes an integral-membrane protein originally found to inhibit apoptosis of hematopoietic cell lines following growth factor deprivation, and later to prevent or delay apoptosis induced by a large variety of stimuli including  $\gamma$ -irradiation, glucocorticoids, heat shock and many chemotherapeutic drugs (2-5). In addition to *Bcl-2*, a structurally related protein *Bcl-x<sub>L</sub>*, has been identified by Craig Thompson's and our laboratory (6-7). *Bcl-x<sub>L</sub>* exhibits different regulation than *Bcl-2*, but like *Bcl-2* inhibits apoptosis induced by a variety of signals (6-11). The biochemical mechanism by which *Bcl-2* and *Bcl-x<sub>L</sub>* block apoptosis is not yet clear. However, *Bcl-2* is known to interact with several cellular proteins (12-17). Some of these proteins, for example Bax, Bad, and Bak, share significant amino acid homology with *Bcl-2* and *Bcl-x<sub>L</sub>* and are considered members of the *Bcl-2* family (18). Bax, Bad and Bak appear to negatively affect *Bcl-2* and/or *Bcl-x<sub>L</sub>* function through protein-protein interactions (13-15). In cancer, one of the most common abnormalities is deregulated expression of the *Bcl-2* oncoprotein. Up to 60% of cancers originating from many organs including breast overexpress a member of the *Bcl-2* family of proteins that inhibits apoptosis (19-23).

A major genetic event that occurs in the genesis and/or progression of breast carcinoma involves alterations in the pathway of apoptosis. We hypothesize that one of these is the functional deregulation of the *Bcl-2* family of proteins and that expression of these proteins plays a critical role in the maintenance of breast cancer cells and resistance of tumor cells to therapy-induced apoptosis. To determine the role of the *Bcl-2* family of proteins in maintaining cancer cell viability, we constructed a recombinant adenovirus vector that expresses *Bcl-x<sub>S</sub>*, a dominant inhibitor of these proteins. Even in the absence of an exogenous apoptotic signal, this recombinant virus specifically and efficiently activates apoptosis in human carcinoma cells arising from multiple organs including breast, colon, stomach and sympathetic nervous tissue (24). Based on these results, we hypothesize that apoptotic signals are constitutively expressed in proliferating cancer cells and perhaps in normal cells, although repressed by members of the *Bcl-2* family of proteins. In this proposal, we proposed studies (i) to determine the mechanism involved in *Bcl-x<sub>S</sub>*-mediated apoptosis using the *bcl-x<sub>S</sub>* adenovirus to dissect molecular interactions of the *Bcl-2* regulatory pathway, (ii) to characterize cellular proteins that interact with *Bcl-x<sub>S</sub>* using biochemical and genetic approaches, and (iii) to use a transgenic model of *Bcl-x<sub>S</sub>* expression in the breast to assess the requirement for *Bcl-2*/*Bcl-x<sub>L</sub>* in the maintenance of normal breast epithelia and tumor growth *in vivo*. The studies outlined in this proposal may provide novel insight into the apoptosis pathway and lead to alternative therapeutic strategies for the treatment of breast cancer and other malignancies.

## BODY OF THE ANNUAL REPORT

### Technical Objective #1: Further characterization of the interaction of Bcl-x<sub>S</sub> with Bcl-2, Bcl-x<sub>L</sub> and Bax in breast cancer cells (Tasks 1.1 and 1.2).

We presented evidence in the original proposal that MCF-7 breast tumor cells and primary breast carcinoma cells undergo apoptotic cell death after exposure to a *bcl-x<sub>S</sub>* adenovirus vector. Our hypothesis is that inactivation of Bcl-2 or Bcl-x<sub>L</sub> by Bcl-x<sub>S</sub> unleashes endogenous death signals leading to execution of the apoptotic program. We proposed experiments to examine molecular interactions of the Bcl-2 family members following expression of Bcl-x<sub>S</sub> protein, but prior to morphological/biochemical cell death, in order to define those interactions which may be created or destroyed as the apoptotic program is activated.

In these experiments, cancer cells that express stably FLAG-tagged Bcl-2 or Bcl-X<sub>L</sub> were infected with the *bcl-x<sub>S</sub>* or control  $\beta$ -galactosidase adenovirus vector. Twenty hours later the infected cells were cultured in media containing <sup>35</sup>S-methionine-cysteine for 4-5 hrs to label newly synthesized proteins in infected cells. Cells were then lysed and protein was immunoprecipitated with anti-FLAG antibody in a buffer containing non-ionic detergents. After immunoprecipitation, the protein complexes were disrupted by heating in the presence of 1% SDS. The supernatant was then diluted and reimmunoprecipitated using anti-Bcl-X or control antibody. Using this approach, it was difficult to detect interactions between Bcl-X<sub>S</sub> and Bcl-2 or Bcl-X<sub>L</sub> due to the low sensitivity of the assay. To overcome this problem, we performed sequential Immunoprecipitation/Western-blot analysis of the same lysates. In this type of experiment, cellular lysates were immunoprecipitated with anti-FLAG and protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Bcl-X antibody. These experiments showed that Bcl-X<sub>S</sub> interacts with Bcl-X<sub>L</sub> but not with Bcl-2.

We determined next if Bcl-X<sub>S</sub> expression could alter the interaction of Bcl-2 or Bcl-X<sub>L</sub> with BAX in SHEP cells. In these experiments, FLAG-tagged-Bcl-2/Bax or Bcl-X<sub>L</sub>/Bax complexes were immunoprecipitated with anti-FLAG in the presence or absence of Bcl-X<sub>S</sub>, and the amount of bound BAX was determined by immunoblotting with anti-BAX antibody. The results showed that Bcl-X<sub>S</sub> displaces BAX from Bcl-2 and Bcl-X<sub>L</sub> after normalization for equal amounts of BAX. We could not determine if Bcl-X<sub>S</sub> could alter the amount of BAK, another partner of Bcl-2 and Bcl-X<sub>L</sub> because the cells did not express detectable levels of endogenous BAK.

Recent experiments have suggested that Bcl-2 and Bcl-X<sub>L</sub> regulate apoptosis by interacting with and inhibiting caspases (death proteases) through a mammalian homologue of the *C. Elegans* CED-4 protein (25-26). Consequently, we are exploring the possibility that Bcl-X<sub>S</sub> promotes apoptosis by interfering with the ability of Bcl-2 and Bcl-X<sub>L</sub> to regulate caspase activity. In preliminary experiments, we have found that Bcl-X<sub>S</sub> can form a multimeric complex with Bcl-X<sub>L</sub> and *C. Elegans* CED-4. Future experiments are aimed at exploring functional and biochemical interactions of Bcl-X<sub>S</sub> with Bcl-X<sub>L</sub>, CED-4 and mammalian caspases.

### Technical Objective #2: Further characterization and purification of p15, a cellular protein that interacts with Bcl-x<sub>S</sub>.

A mechanism that might explain the apoptosis-promoting activity of Bcl-x<sub>S</sub> is through binding to an upstream activator or a downstream effector of Bcl-2/Bcl-x<sub>L</sub> mediated survival. In preliminary



results, we provided evidence in the original application that Bcl-x<sub>S</sub> interacts with a cellular protein, p15, in cancer cells infected with the *bcl-x<sub>S</sub>* adenovirus. The significance of the Bcl-x<sub>S</sub>-p15 interaction was unclear. p15 could represent a death effector which is activated by the expression of Bcl-x<sub>S</sub>. Alternatively, p15 could be a cellular protein required for survival whose activity is inhibited by the Bcl-x<sub>S</sub> interaction. Clearly, biochemical characterization of p15 and isolation of the p15 cDNA was needed to further assess the role of p15 in Bcl-x<sub>S</sub>-mediated apoptosis.

Biochemical Purification and N-Terminal Microsequencing of p15 (Task 2.1) We purified p15 from preparative gels and submitted the material for microsequencing. Sequencing analysis revealed that the N-terminal sequence of p15 is identical to that of a region of Bcl-2, amino acids that are part of the flexible loop region (27). Thus, p15 represents a cleavage product of Bcl-2. Given these results we are not pursuing any longer the characterization of the protein band termed p15.

Genetic Screen for Bcl-x-Binding Proteins using the Two-Hybrid Yeast Assay (Task 2.2). To search for Bcl-x-interacting proteins, we screened placenta and brain cDNA libraries using Gal4-Bcl-x<sub>L</sub> and Gal4-Bcl-x<sub>S</sub> as "baits" in the yeast two-hybrid assay. In the first screen using the GAL4-Bcl-x<sub>L</sub> bait, fifty six positive clones were identified that interacted with Gal4-Bcl-x<sub>L</sub>. Forty one cDNAs encoded Bad, a Bcl-2 family member recently isolated by binding to Bcl-2. Ten cDNAs encoded Bcl-2 which is known to bind Bcl-X<sub>L</sub> in the two-hybrid system. The nucleotide sequences of four cDNAs were novel in that they did not reveal significant homology to any known gene or translated products in the databases. Three of these novel cDNAs encoded the same gene which we have named *harakiri*. The same gene was identified as an interacting partner of Bcl-2 (28). *Harakiri* functions as a regulator of Bcl-2 and Bcl-X<sub>L</sub> and apoptotic cell death in mammalian cells. The *hrk* product (HRK) does not exhibit significant homology to Bcl-2 or Bcl-X<sub>L</sub> and lacks the conserved BH1 and BH2 domains which are shared by Bcl-2 family members. Significantly, HRK physically interacts with anti-apoptosis proteins Bcl-2 and Bcl-X<sub>L</sub> but not with death-promoting Bcl-2 members such as Bax and Bak. Expression of HRK induces rapid onset of cell death in mammalian cells including breast cancer cells. Importantly, the death-promoting activity of HRK is repressed by Bcl-2 and Bcl-X<sub>L</sub> suggesting that HRK is a common target for the anti-apoptosis proteins Bcl-2 and Bcl-X<sub>L</sub>. Details of these results have been recently published (Inohara *et al.* The EMBO J. 16:1686-1694, 1997).

Technical Objective #3: Characterization of Transgenic Mice Expressing Inducible Bcl-x<sub>S</sub> in the Breast (Task 3.1).

The studies described above suggest that Bcl-2, Bcl-X<sub>L</sub> or a related protein that is inhibited by Bcl-x<sub>S</sub> is required for viability of epithelial cancer cells in culture. It is unclear, however, whether the survival of normal epithelial cells or primary carcinomas can be inhibited by Bcl-x<sub>S</sub> *in vivo*. To begin to address these questions, development of a transgenic mouse model of Bcl-x<sub>S</sub> expression in the breast was proposed in the original application.

Because Bcl-x<sub>S</sub> may induce cell death in normal breast epithelium *in vivo*, an inducible, tissue-specific system was proposed. In this system, a tetracycline-controlled trans-activator (tTA), composed of the repressor of the tetracycline-resistance operon (*Teto* from *Escherichia coli* transposon Tn10) and the activating domain of viral VP16 of herpes simplex virus regulate gene expression from a minimal cytomegalovirus (mCMV) promoter containing seven *tet* operator sequences (29). In the presence of tetracycline, transcription of mCMV-driven genes is repressed. However, in the absence of tetracycline, the tTA binds to the tet operator sequences which leads to strong transcriptional activation of the CMV promoter (29). The *bcl-x<sub>S</sub>* cDNA was inserted into the

minimal hCMV (human cytomegalovirus) cassette (a gift of Dr. Hermann Bujard, University of Heidelberg). The recombinant hCMV-*bcl-x<sub>S</sub>* plasmid was microinjected into mouse zygotes at the University of Michigan Transgenic Core Facility. As a control, recombinant plasmid expressing hCMV-*bcl-x<sub>L</sub>* was also created and microinjected into mouse zygotes. Five *bcl-x<sub>S</sub>* transgenic founder mice that expressed the transgene were identified by analysis of tail genomic DNA. We generated double *bcl-x<sub>S</sub>/tTA* transgenic mice by crossing a mouse from each *bcl-x<sub>S</sub>* founder line with a transgenic mouse carrying tTA under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (a gift of Dr. Priscilla Furth, the University of Maryland). Analysis of breast and salivary gland tissues from double transgenic female mice revealed that the mice did not express detectable Bcl-X<sub>S</sub> protein in the absence of tetracycline. Given these negative results, we generated another set of transgenic mouse lines in which Bcl-X<sub>S</sub> was targeted to the breast by the WAP promoter (30). We obtained 17 independent transgenic WAP-Bcl-X<sub>S</sub> lines as determined by PCR analysis of founder mice. Of these founder mice, 13 out of the 17 passed the WAP-Bcl-X<sub>S</sub> transgene to their progeny. We performed expression analysis of the Bcl-X<sub>S</sub> transgene in lactating breast at a time in which the WAP promoter is known to be active and induce expression of transgenes. Western blot analysis revealed that the Bcl-X<sub>S</sub> transgene was expressed in the lactating breast in 2 out of 13 WAP-Bcl-X<sub>S</sub> transgene mouse lines. Histological analysis of the breast tissue on day 10 of lactation after the first pregnancy from positive WAP-Bcl-X<sub>S</sub> lines showed normal histology when compared to controls. However, follow-up analysis after the second pregnancy revealed abnormal histology in the breast from WAP-Bcl-X<sub>S</sub> transgenic females. Initial studies indicate that the mammary tissue from WAP-Bcl-X<sub>S</sub> transgenic females failed to undergo pregnancy-induced differentiation despite normal pregnancy and birth of pups. The mechanism responsible for the phenotype observed in WAP-Bcl-X<sub>S</sub> transgenic mice is under investigation.



## CONCLUSIONS

The studies which we have performed in the past year are important in that they have provided information regarding the mechanism by which the Bcl-X<sub>S</sub> adenovirus kills tumor cells. Particularly revealing was the interaction of Bcl-X<sub>S</sub> with Bcl-X<sub>L</sub> but not with Bcl-2 suggesting that Bcl-X<sub>S</sub> functions at least in part through another regulator. A candidate for this regulator is a mammalian homologue of the *C. Elegans* protein CED-4. Through a genetic screen in yeast, we have identified and characterized HRK, a novel protein that interacts with Bcl-2 and Bcl-X<sub>L</sub> and induces apoptosis of cancer cell lines including breast tumor cells. Another observation during the past year has been that expression of a Bcl-X<sub>S</sub> transgene in the breast resulted in the loss of pregnancy-induced differentiation of the mammary epithelium. Although the mechanism of the phenotype remains unclear, these results suggest that proteins of the Bcl-2 survival pathway that are targeted by Bcl-X<sub>S</sub> may be required for the terminal differentiation of mammary epithelium induced by pregnancy.

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# ***harakiri*, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X<sub>L</sub>**

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Programmed cell death is essential in organ development and tissue homeostasis and its deregulation is associated with the development of several diseases in mice and humans. The precise mechanisms that control cell death have not been elucidated fully, but it is well established that this form of cellular demise is regulated by a genetic program which is activated in the dying cell. Here we report the identification, cloning and characterization of *harakiri*, a novel gene that regulates apoptosis. The product of *harakiri*, Hrk, physically interacts with the death-repressor proteins Bcl-2 and Bcl-X<sub>L</sub>, but not with death-promoting homologs, Bax or Bak. Hrk lacks conserved BH1 and BH2 regions and significant homology to Bcl-2 family members or any other protein, except for a stretch of eight amino acids that exhibits high homology with BH3 regions. Expression of Hrk induces cell death which is inhibited by Bcl-2 and Bcl-X<sub>L</sub>. Deletion of 16 amino acids including the conserved BH3 region abolished the ability of Hrk to interact with Bcl-2 and Bcl-X<sub>L</sub> in mammalian cells. Moreover, the killing activity of this mutant form of Hrk (Hrk ΔBH3) was eliminated or dramatically reduced, suggesting that Hrk activates cell death at least in part by interacting with and inhibiting the protection afforded by Bcl-2 and Bcl-X<sub>L</sub>. Because Hrk lacks conserved BH1 and BH2 domains that define Bcl-2 family members, we propose that Hrk and Bik/Nbk, another BH3-containing protein that activates apoptosis, represent a novel class of proteins that regulate apoptosis by interacting selectively with survival-promoting Bcl-2 and Bcl-X<sub>L</sub>.

**Keywords:** apoptosis/Bcl-2 family/Hrk/programmed cell death

## **Introduction**

Apoptosis, a morphologically distinguished form of programmed cell death, is critical not only during development and tissue homeostasis but also in the pathogenesis of a variety of diseases including cancer, autoimmune disease, viral infection and neurodegenerative disorders (Thompson, 1995). The precise mechanisms that regulate apoptosis have not been elucidated; however, it appears that this form of cell death is controlled by a genetic program which is activated in the dying cell (Thompson, 1995;

White, 1996). Several regulatory components of the apoptotic pathway have been identified in various living organisms including man (Thompson, 1995; White, 1996). *bcl-2*, the first member of an evolutionarily conserved family of apoptosis regulatory genes, was isolated initially from the t(14;18) chromosomal translocation found in human B-cell follicular lymphomas and subsequently was shown to repress cell death triggered by a diverse array of stimuli (Vaux *et al.*, 1988; Núñez *et al.*, 1990; Strasser *et al.*, 1991). Several members of the family including Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, A1, Bcl-w and Ced-9 share conserved regions termed Bcl-2 homology domain 1, 2, 3 and 4 (BH1, BH2, BH3 and BH4) and function by repressing apoptosis (for review, see White, 1996). In contrast, other members of the family, such as Bax and Bak, share BH1, BH2 and BH3 domains and regulate apoptosis by promoting cell death at least in part through physical interactions with death-repressing Bcl-2 family members (Oltvai and Korsmeyer, 1994; Sato *et al.*, 1994).

Two members of the family, Bcl-2 and Bcl-X<sub>L</sub>, are expressed in many embryonic and adult tissues, and their importance has been revealed by analysis of mutant mice deficient in Bcl-2 or Bcl-X<sub>L</sub>. Null mutations of *bcl-x* are embryonic lethal with the occurrence of profound cell death, whereas mice deficient in *bcl-2* die early after birth (Veis *et al.*, 1993; Motoyama *et al.*, 1995). However, the biochemical process by which Bcl-2 and Bcl-X<sub>L</sub> repress cell death and the mechanisms that regulate their functions are poorly understood. The susceptibility of a cell to apoptotic signals appears to be regulated in part by the relative levels and competing dimerizations of different Bcl-2 family members (Oltvai and Korsmeyer, 1994). In addition to Bcl-2 family members, Bcl-2 is known to interact with several cellular proteins that include R-Ras, Nip proteins and BAG-1 which appear to be part of signal transduction pathways (Boyd *et al.*, 1994; Fernandez-Sarabia and Bischoff, 1994; Takayama *et al.*, 1995; Wang *et al.*, 1996). However, the biochemical significance of these Bcl-2 interactions is unclear and poorly understood.

Here we report the identification, cloning and characterization of *harakiri*, a novel apoptosis regulatory gene. The product of *harakiri*, Hrk, physically interacts with the death-repressor proteins Bcl-2 and Bcl-X<sub>L</sub>, but not with death-promoting Bax or Bak. Hrk lacks conserved BH1 and BH2 regions and significant homology to Bcl-2 family members or any other protein, except for a stretch of eight amino acids that exhibits high homology with BH3 regions. Hrk structurally resembles Bik/Nbk, another BH3-containing protein which was identified recently as an interacting partner of the adenovirus E1B 19K protein and Bcl-2 (Boyd *et al.*, 1995; Han *et al.*, 1996b). Beyond the conserved BH3 region, however, Bik/Nbk and Hrk do not share any significant amino acid homology. Expression of Hrk induced cell death which was repressed by Bcl-2 and

Bcl-X<sub>L</sub>. Deletion of the conserved BH3 region in Hrk abolished its ability to interact with Bcl-2 and Bcl-X<sub>L</sub>, and dramatically diminished its killing activity when compared with wild-type Hrk. Because Hrk lacks conserved BH1 and BH2 domains that define Bcl-2 family members, we propose that Bik/Nbk and Hrk represent a novel class of proteins that regulate apoptosis at least in part by interacting selectively with survival-promoting Bcl-2 and Bcl-X<sub>L</sub>.

## Results

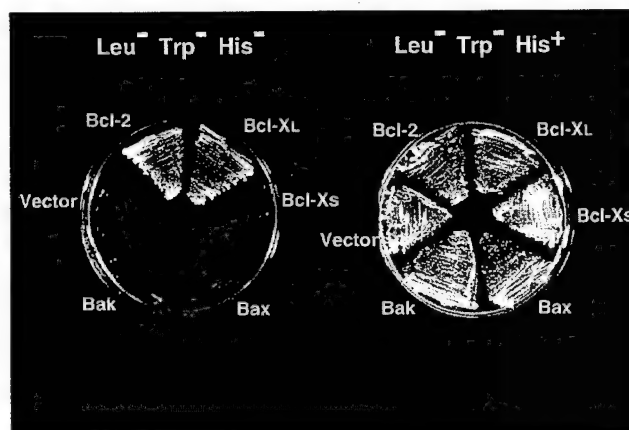
### **Two-hybrid screening for proteins that bind to Bcl-2 reveals a novel protein that interacts with anti-apoptosis proteins Bcl-2 and Bcl-X<sub>L</sub> but not with death-promoting proteins Bax and Bak**

To search for Bcl-2-interacting proteins, we screened a HeLa cDNA library using GAL4-Bcl-2 as a 'bait' in the yeast two-hybrid assay. In a screen of  $3 \times 10^6$  library clones, 30 positive clones were identified that interacted with the GAL4-Bcl-2 bait. Plasmids recovered from the original yeast strain were used in a co-transformation assay with the original GAL4-Bcl-2 bait or control heterologous baits to discard false-positive clones. Twenty one cDNAs (designated BP1-21) were found to contain inserts of different sizes (from 1.3 to 0.7 kb) that interacted with the GAL4-Bcl-2 bait but not with control baits. These cDNA clones were characterized by restriction enzyme mapping and nucleotide sequence analysis. As expected, multiple clones encoded proteins which are known to associate with Bcl-2 in mammalian cells. Seven cDNAs encoded Bad, three encoded Bax and two encoded Bik/Nbk (Oltvai *et al.*, 1993; Boyd *et al.*, 1995; Yang *et al.*, 1995; Han *et al.*, 1996b). Four cDNAs encoded a Ras-related protein (Yamagata *et al.*, 1994). The two-hybrid screen also yielded five cDNA clones with novel nucleotide sequences. Restriction enzyme and sequence analysis revealed that four of the novel positive clones contained identical inserts of 712 bp fused to the GAL4 DNA-binding domain. One of these four cDNA clones was characterized further. We have named this novel gene 'harakiri' and the product that it encodes Hrk, after the Japanese suicide ritual (see below).

To characterize the specificity of the Hrk interactions further, we determined the ability of Hrk to interact with Bax, Bak and Bcl-X<sub>S</sub>, three Bcl-2-related proteins that promote cell death. In the yeast two-hybrid system, Hrk interacted with Bcl-2 and Bcl-X<sub>L</sub> but not with Bax, Bak or Bcl-X<sub>S</sub> (Figure 1). These results indicate that Hrk selectively interacts with Bcl-2 family members such as Bcl-2 and Bcl-X<sub>L</sub> which are functionally related in that they are capable of inhibiting apoptosis. Importantly, Hrk failed to associate with Bcl-X<sub>S</sub>, an alternative form of Bcl-X, that lacks an internal region of 62 amino acids that is required for Bcl-X<sub>L</sub> protective function (Boise *et al.*, 1993).

### **Hrk interacts with Bcl-2 and Bcl-X<sub>L</sub> in mammalian cells**

To confirm that Hrk associates with Bcl-2 and Bcl-X<sub>L</sub> in mammalian cells, a 293T human kidney cell line was transiently co-transfected with expression plasmids producing a Flag-tagged Hrk protein and Bcl-2, Bcl-X<sub>L</sub> or

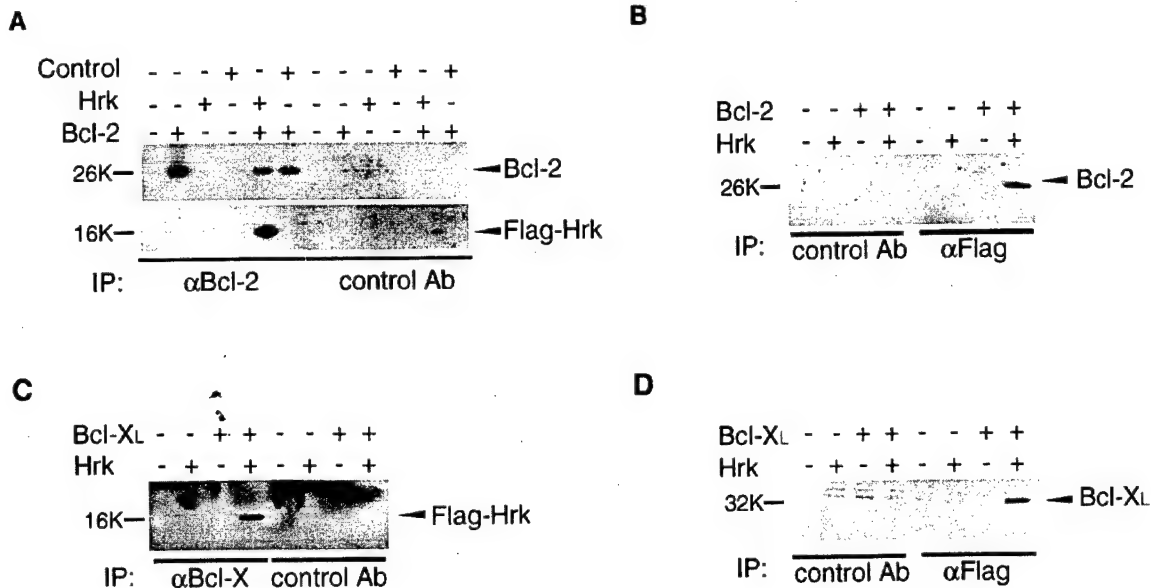


**Fig. 1.** Specificity of the interaction between Hrk and Bcl-2 family members in yeast. A plasmid expressing Hrk fused to the GAL4 DNA-binding domain was co-transfected with plasmids expressing Bcl-2 family members fused to the GAL4 transcriptional activation domain (AD). Growth of yeast in the absence of leucine, tryptophan and histidine is indicative of protein-protein interaction. Growth in the absence of leucine and tryptophan is shown as a control.

control plasmid. In these initial experiments, the entire *harakiri* sequence that was fused to the GAL4 DNA-binding domain was cloned into expression plasmids to produce Flag-Hrk. Immunoprecipitates were prepared using hamster anti-Bcl-2 monoclonal antibody and subjected to SDS-PAGE and immunoblotting using anti-Flag or Bcl-2 antibody. Immunoblotting with anti-Bcl-2 antibody revealed that Flag-Hrk was co-immunoprecipitated with Bcl-2 (Figure 2A). Control immunoblotting with anti-Bcl-2 antibody confirmed that Bcl-2 was immunoprecipitated in lysates of cells transfected with the *bcl-2* plasmid (Figure 2A). The Hrk-Bcl-2 interaction was specific in that it required co-expression of both Bcl-2 and Flag-Hrk, and was not detected when the lysate was immunoprecipitated with control antibody (Figure 2A). To verify the interaction, we performed reciprocal experiments using anti-Flag antibody to immunoprecipitate Flag-Hrk, followed by Western blot with anti-Bcl-2 antibody. In agreement with the reverse experiment, Bcl-2 co-immunoprecipitated specifically with Flag-Hrk (Figure 2B).

To determine if Hrk associates with Bcl-X<sub>L</sub> in mammalian cells, we performed immunoprecipitation experiments similar to those performed to assess the Hrk-Bcl-2 interaction. After transfection of 293T cells with expression plasmids producing a Flag-tagged Hrk protein and Bcl-X<sub>L</sub> or control plasmid, immunoprecipitates were prepared using anti-Bcl-X antibody and subjected to SDS-PAGE immunoblotting using anti-Flag antibody. Importantly, Flag-Hrk was co-immunoprecipitated specifically with Bcl-X<sub>L</sub> (Figure 2C). To confirm the interaction, we performed reciprocal experiments using anti-Flag antibody to immunoprecipitate Flag-Hrk, followed by Western blot with anti-Bcl-X antibody. In agreement with the reverse experiment, Bcl-X<sub>L</sub> co-immunoprecipitated specifically with Flag-Hrk (Figure 2D). We also tested if Hrk interacts with Bax in 293T cells and found that Flag-Hrk failed to co-immunoprecipitate with Bax, confirming our results in the yeast assay (data not shown).





**Fig. 2.** Hrk interacts with Bcl-2 and Bcl-X<sub>L</sub> in mammalian cells. 293T cells ( $5 \times 10^6$  per 100 mm plate) were transiently transfected with 10  $\mu$ g of the indicated plasmids, pSFFV-Flag-tagged BP25 (control), pSFFV-Flag-Hrk16K (Hrk), pSFFV-hBcl-2 (Bcl-2), pSFFV-HA-hBcl-X<sub>L</sub> (Bcl-X<sub>L</sub>) or pSFFV-HA-mBax (Bax). In the case of transfection with a single plasmid, cells were co-transfected with 10  $\mu$ g of empty pSFFV-neo vector and the total amount of plasmid DNA was always 20  $\mu$ g. (A) Lysates were immunoprecipitated with anti-Bcl-2 or control antibody. (B) Anti-Flag or control antibody immunoprecipitates were immunoblotted with anti-Bcl-2 antibody. (C) Anti-Bcl-X or control antibody immunoprecipitates were immunoblotted with anti-Flag antibody. (D) Anti-Flag or control antibody immunoprecipitates were immunoblotted with anti-Bcl-X antibody. Notice that pSFFV-Flag-Hrk16K produces 16 kDa protein, as the entire *harakiri* cDNA including its 5' untranslated region was fused in-frame with the Flag sequence and the amino-terminus of Hrk.

#### ***harakiri* encodes a novel protein with a conserved BH3 region but lacking BH1 or BH2 domains**

To confirm the sequence obtained by the two-hybrid assay, a 9-week embryo human cDNA library was screened by hybridization with a labeled *harakiri* probe. Forty positive cDNA clones were identified and characterized by restriction mapping, PCR analysis and sequencing. Analysis of the longest cDNAs revealed inserts of 716 bp essentially identical in sequence to that observed previously fused to the GAL4 DNA-binding domain. A single nucleotide difference C→T at position 334 of the *harakiri* cDNA was noted between the coding sequences obtained from the HeLa and embryo cDNA libraries (Figure 3A). This nucleotide difference did not change the amino acid sequence of Hrk, reflecting perhaps a gene polymorphism. Northern blot analysis of mouse and human tissues using a labeled *harakiri* probe identified a major mRNA species of ~0.7 kb in certain tissues (see below). Taken together, these results suggest that the isolated cDNA clones are full-length. The cDNAs encode an open reading frame of 91 amino acids (Figure 3A). In the 3' untranslated region, an ATTTA sequence motif for RNA destabilization (Shaw and Kamen, 1986) and a poly(A) tail were identified. Initial DNA searches using the NCBI BLAST program revealed that the nucleotide sequence was novel in that they did not reveal significant homology to any known gene or translated products in the databases. However, close inspection of the Hrk protein revealed a stretch of eight amino acids, Hrk 37–44, with high homology to a BH3 motif. This conserved region is shared by Bcl-2 family members (Chittenden et al., 1995b; Zha et al., 1996). The BH3 region of Hrk was contained within a predicted  $\alpha$ -helix which has been described recently in the crystal structure of Bcl-X<sub>L</sub> (Muchmore et al., 1996),

and predicted to be present in other Bcl-2 family members (Figure 3C). A region of 28 hydrophobic residues that may serve as a membrane-spanning domain was identified at the COOH-terminus of the Hrk coding region (Figure 3A).

#### ***harakiri* displays a highly restricted expression in human tissues**

To characterize *harakiri* further, its expression pattern was examined by Northern blot analysis using a *harakiri* probe of various RNA samples isolated from human adult tissues. A major 0.7 kb transcript was detected in all lymphoid tissues tested but was expressed predominantly in bone marrow and spleen (Figure 4). Further analysis showed that *harakiri* mRNA was also expressed in pancreas and at very low or undetectable levels in kidney, liver, lung and brain (data not shown).

#### **The Flag-Hrk protein is a non-nuclear intracellular protein**

In order to begin to assess the subcellular localization of Hrk, we transiently transfected a Flag-*harakiri* construct into 293 cells and determined the intracellular localization of the Flag-Hrk protein by laser scanning confocal microscopy. Analysis of labeled cells with anti-Flag monoclonal antibody revealed that Flag-Hrk displays a labeling pattern which was granular and extranuclear, consistent with a localization confined to membranes of intracellular organelles (Figure 5A). The labeling pattern of Hrk was similar to that previously reported for Bcl-2 and Bcl-X<sub>L</sub>, suggesting that Hrk, Bcl-2 and Bcl-X<sub>L</sub> localize to similar intracellular compartments in mammalian cells (Krajewski et al., 1993; González-García et al., 1994). The specificity of the labeling was determined by assessing the staining

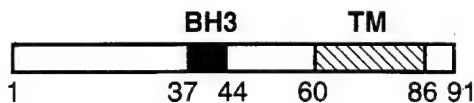
A

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GAAACTTGGTGTCCAGGGAGGCCCGCGCGGTGGAGCGCGCGCGAGCGCGCGAGAG 60
GCCGAGGAGGAGGAGGCGAGGGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 120
ATGTGCGCGTGGCGCGTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 180
M C P C P L H R G R G P P A V C A C S A 20
GGTGGCGTGGCGCGTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 240
G R L G L R S S A A Q L T A A R L K A L 40
GGCGAGAGTGCACCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 300
G D E L H Q R T M W R R R A R S R R A P 60
GGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 360
A P G A L P T Y W P W L C A A A Q V A A 80
CTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 420
L A A W L L G R R N L 91
CGGAGCGAGACCCAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 480
AAGCTGGTTCGCGTTTCCAGAGGGGAGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 540
CGAGAGGAGTGGAGAGTAAAGACAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 600
CTGGGGAAGCCCTTGGAAATCCAGCTGCAGAAACAGACACCCCAATGCTATTTCATAC 660
AGCTCTATATATATAAAAAAGAAATATGAATATTAATAAAAAAATAAAAAA 716

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B



C

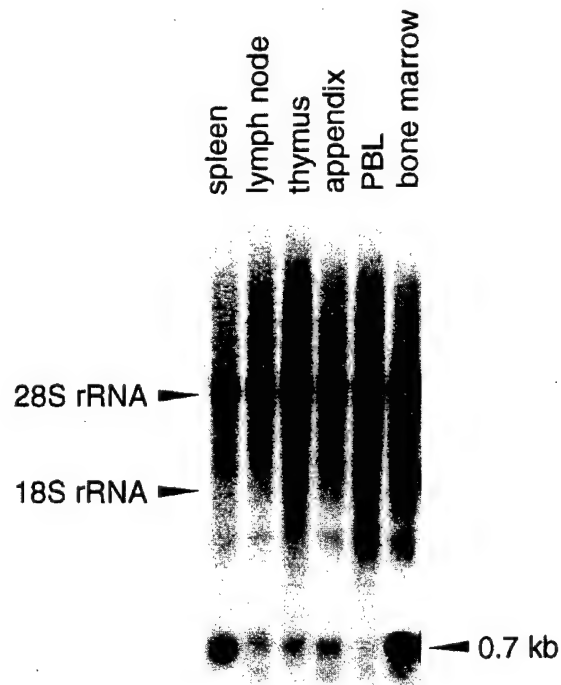
		BH3	
Hrk	31	QLTAARLKALGDELHQRTMWRRR	53
Bax	57	KKLSECLKRIGDELDSNMELQRM	79
Bcl-2	88	PVVLALRQAGDDFSRRYRGDFA	110
Bcl-x	84	AAVKQALREAGDEFELRYRAFS	106
Bik/Nbk	55	DALALRLACIGDEMDVSLRAPRL	77
Bak	72	GQVGRQLAIIGDINRRYDSEFQ	94
Mcl-1	207	RKALETLLRRVGDGVQRNHETVFQ	229

**Fig. 3.** *harakiri* encodes a novel BH3-containing protein. (A) The nucleotide sequence of human *harakiri* cDNA. The sequence from the longest cDNA clone isolated from a 9-week embryo is shown. The coding region is indicated with its amino acid sequence. A conserved BH3 region and a putative transmembrane domain are indicated by double and single underlining in the amino acid sequence, respectively. An ATTTA sequence motif for RNA destabilization identified in the 3' untranslated region is boxed. A putative nucleotide polymorphism was observed, C→T at position 334 of the cDNA, and is shown by underlining in the nucleotide sequence. (B) Schematic structure of Hrk. (C) Comparison of the BH3 domains between Hrk and Bcl-2 family members. Identical and conserved hydrophobic residues are indicated by bold letters and stars, respectively. References for hBax, hBcl-2, hBcl-X, Bik/Nbk, Bak and Mcl-1 proteins are given in the Introduction.

pattern obtained after transfection with a control Flag-tagged GATA-1 expression plasmid. Staining of the GATA-1 protein with anti-Flag antibody revealed a nuclear labeling pattern, as expected for a nuclear transcriptional factor (Figure 5B).

#### Expression of Hrk induces rapid cell death which is repressed by Bcl-2 and Bcl-X<sub>L</sub>

The effect of *harakiri* on cell survival was examined initially in 293T cells using a transient transfection assay. Transfection of a *harakiri* expression plasmid into 293T cells resulted in a dramatic loss of cell viability at 36 h post-transfection (Figure 6B and F). This was specific in that transfection of 293T cells with empty vector, *bcl-2* or *bcl-x<sub>L</sub>* expression plasmids did not have any effect on



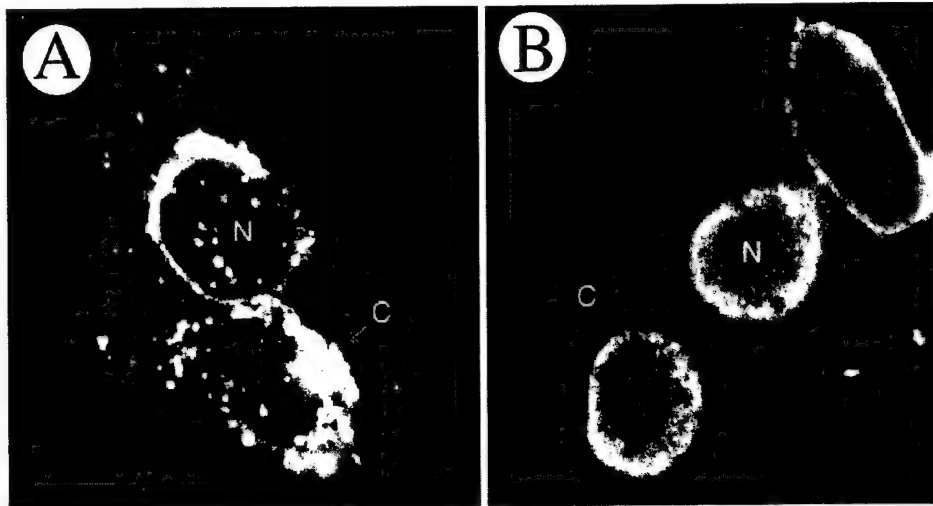
**Fig. 4.** Expression of *harakiri* mRNA by Northern blot analysis. A filter loaded with 2 µg of poly(A) RNA from various tissues of the immune system was hybridized with the entire *harakiri* cDNA probe labeled with <sup>32</sup>P, washed with 0.2× SSC at 65°C for 1 h. The filter was exposed to X-ray film for 12 h. 28S rRNA, 18S rRNA and the major transcript of 0.7 kb are indicated by arrowheads. PBL, peripheral blood leukocytes.

cell survival (Figure 6A, C, E and G). Significantly, co-expression of Bcl-2 or Bcl-X<sub>L</sub> inhibited the death-promoting activity of Hrk (Figure 6D and H). To verify these results further, we performed additional experiments in which Hrk plasmids were transfected into 293, HeLa and FL5.12 progenitor B cells. Transient transfection of 293 and HeLa cells with Hrk plasmids induced cell death with kinetics similar to those observed with 293T cells (data not shown). To date, we have been unable to generate any stable FL5.12 cell lines that express transfected Hrk in the absence of exogenous Bcl-2 or Bcl-X<sub>L</sub>, suggesting that Hrk expression is also lethal to these cells (data not shown).

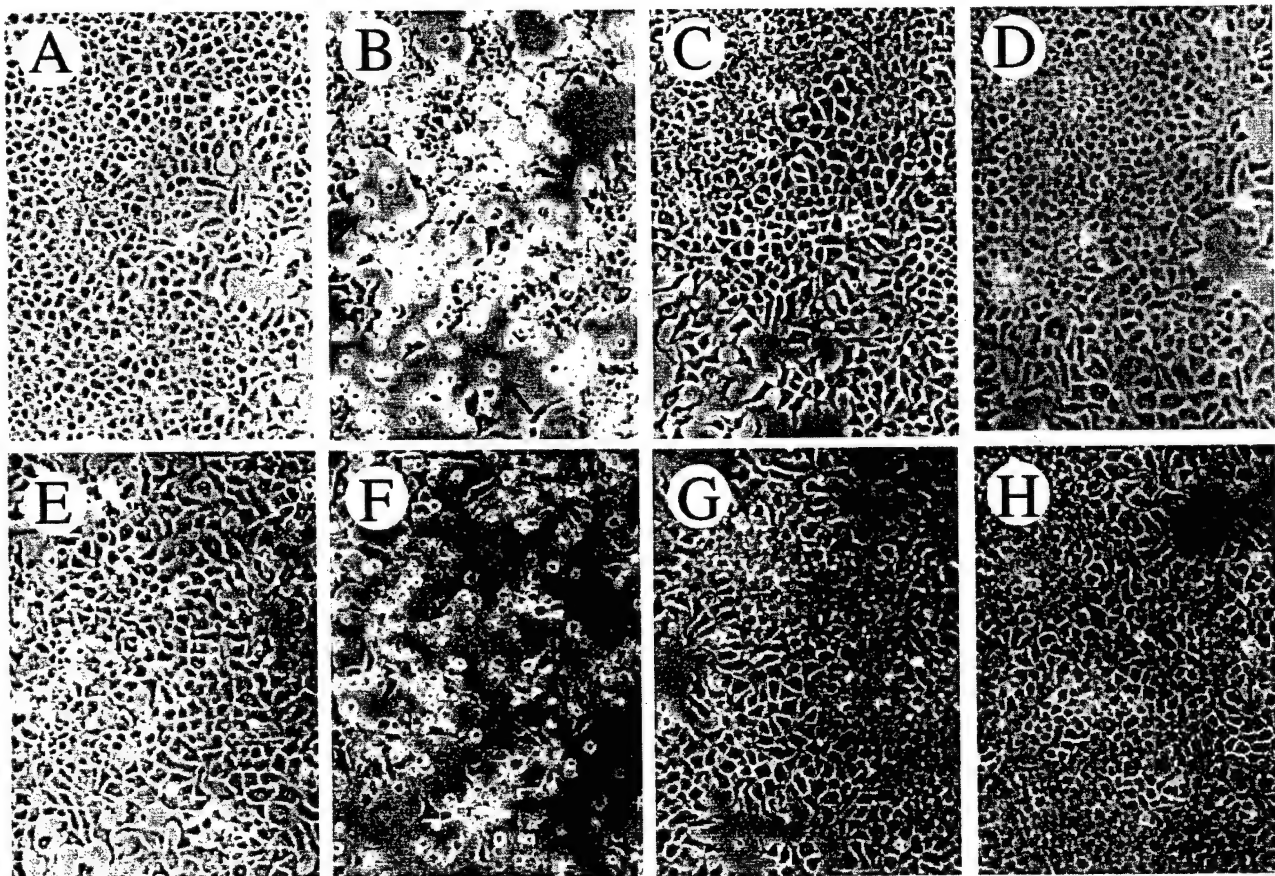
#### Hrk requires a region of 16 amino acids containing BH3 to interact with Bcl-2 and Bcl-X<sub>L</sub>

To determine the ability of Hrk to associate with Bcl-2 or Bcl-X<sub>L</sub> through BH3, we engineered a mutant form of Hrk lacking BH3. A deletion mutant was designed to eliminate residues 34–49 of Hrk, a predicted α-helix, to minimize secondary effects on folding of the Hrk protein. The deleted residues contained the most conserved amino acids of the BH3 homology region of Hrk (Figures 3C and 7A). Expression plasmids producing wild-type or mutant Flag-tagged Hrk and Bcl-2 or Bcl-X<sub>L</sub> were transiently co-transfected into 293T cells. Immunoprecipitates were prepared using anti-Flag to recognize wild-type and mutant Flag-Hrk and subjected to SDS-PAGE and immunoblotting using anti-Bcl-2 and anti-Bcl-X antibodies. Deletion of residues 34–49 (Hrk mutant ΔBH3) completely eliminated the ability of Hrk to interact with





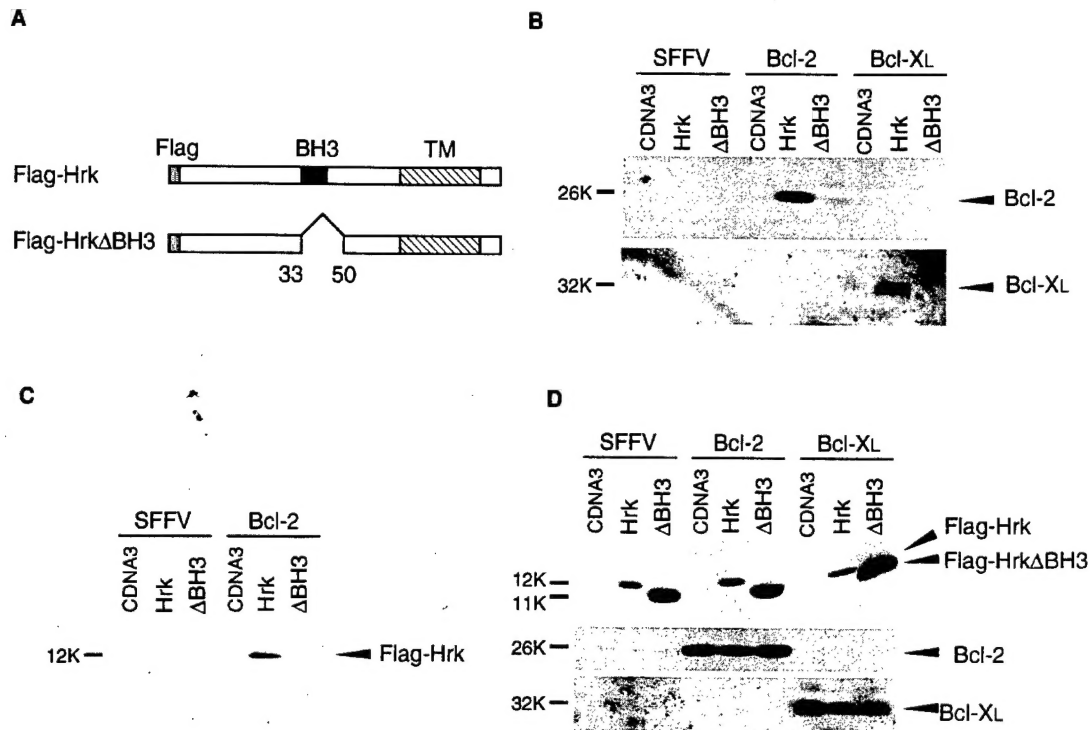
**Fig. 5.** Flag-Hrk protein is a non-nuclear intracellular protein. 293 cells ( $5 \times 10^6$  cells per 100 mm plate) were transfected with 10  $\mu$ g of pSFFV-Flag-Hrk16K (A) or Flag-GATA-1 (B) as a control. Shown are confocal images after labeling with anti-Flag and secondary fluorescein-conjugated antibody. Samples were prepared at 18 h after transfection. Nuclei and cytoplasm are indicated by N and C, respectively. Notice that cells transfected with pSFFV-Flag-Hrk16K round up. Scattered bright particles probably represent cellular fragments from dying neighboring cells.



**Fig. 6.** Expression of Hrk induces cell death which is repressed by Bcl-2 or Bcl- $X_L$ . 293T cells ( $5 \times 10^6$  per 100 mm plate) were co-transfected with 20  $\mu$ g of pSFFV-neo (A and E), 10  $\mu$ g of pSFFV-Flag-Hrk16K plus 10  $\mu$ g of pSFFV-neo (B and F), 10  $\mu$ g of pSFFV-hBcl-2 plus 10  $\mu$ g of pSFFV-neo (C), 10  $\mu$ g of pSFFV-Flag-Hrk16K plus 10  $\mu$ g of pSFFV-hBcl-2 (D), 10  $\mu$ g of pSFFV-HA-hBcl- $X_L$  and 10  $\mu$ g of pSFFV-neo (G), 10  $\mu$ g of pSFFV-Flag-Hrk16K plus 10  $\mu$ g of pSFFV-HA-hBcl- $X_L$  (H) as described in Figure 2. Photographs represent cells at 36 h after transfection. Notice that cultures transfected with Flag-harakiri show a paucity of attached live cells and shrunken dead cells (arrows) which are blocked by co-transfection with bcl-2 or bcl- $x_L$ .

Bcl-2 and Bcl- $X_L$  (Figure 7B). To confirm these results, we performed reciprocal experiments using anti-Bcl-2 antibody, followed by Western blot with anti-Flag antibody.

In agreement with the reverse experiment, wild-type Hrk but not mutant Hrk co-immunoprecipitated specifically with Bcl-2 (Figure 7C). Analysis of total lysates from the



**Fig. 7.** Hrk requires a region of 16 amino acids containing BH3 to interact with Bcl-2 and Bcl-XL. (A) Schematic structure of wild-type (Flag-Hrk) and mutant (Flag-Hrk ΔBH3) proteins. (B) 293T cells ( $5 \times 10^6$  per 100 mm plate) were co-transfected with 2 μg of pCDNA3 (CDNA3), pCDNA3-Flag-Hrk (Hrk) or pCDNA3-Flag-Hrk ΔBH3 (Flag-Hrk ΔBH3) and 10 μg of pSFFV-neo (SFFV), pSFFV-hBcl-2 (Bcl-2) or pSFFV-HA-hBcl-XL (Bcl-XL) as described in Figure 1. Anti-Flag immunoprecipitates were immunoblotted by anti-Bcl-2 (upper) and anti-Bcl-X antibody (lower). (C) Anti-Bcl-2 immunoprecipitates were immunoblotted with anti-Flag antibody. (D) Twenty μg of total lysate were immunoblotted with anti-Flag, anti-Bcl-2 or anti-Bcl-X antibody.

same cellular extracts by immunoblotting confirmed that 293T cells transfected with the corresponding plasmids expressed Bcl-2, Bcl-XL, wild-type and mutant Hrk proteins (Figure 7D).

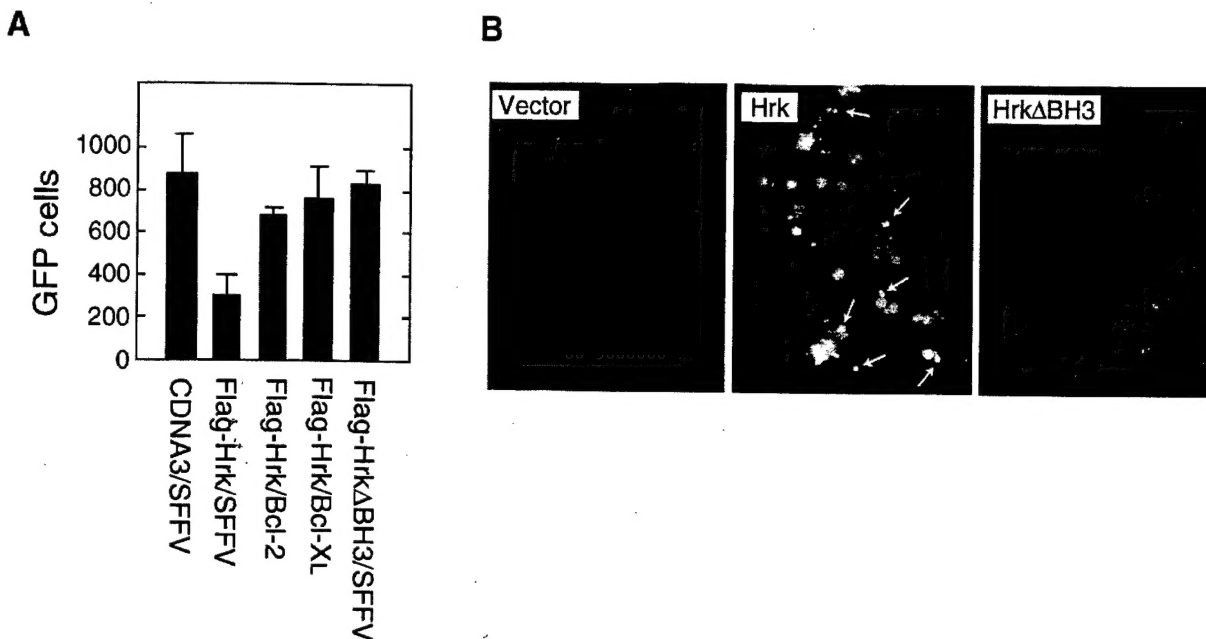
#### The BH3 domain of Hrk is required for the induction of cell death

To determine if the BH3 domain of Hrk is necessary to induce cell death, we compared the killing activity of wild-type and mutant Hrk proteins by a transient transfection assay in 293T cells. The cells were co-transfected with a reporter plasmid expressing GFP (green fluorescence protein), in combination with either an expression plasmid encoding Hrk proteins or control plasmids. The cell killing activity of wild-type and mutant Hrk was measured by a reduction in the number of cells that express the reporter GFP protein relative to that obtained by transfection with a control expression plasmid. The results of these experiments showed that deletion of residues 34–49 of Hrk (Hrk mutant ΔBH3) dramatically reduced the ability of Hrk to kill 293T cells, when compared with the activity exhibited by wild-type Hrk (Figure 8A). Furthermore, expression of Bcl-2 or Bcl-XL inhibited the killing activity of wild-type Hrk, confirming the results presented in Figure 6. The loss of viability and the morphology of the cells observed after transfection with Hrk plasmids in Figure 6 suggested that Hrk may induce apoptotic cell death. To determine if the cell death activated by transient Hrk expression was caused by apoptosis, the nuclei of 293T cells were stained with acridine orange and ethidium bromide. Cells transfected with wild-type Hrk but not

with mutant Hrk ΔBH3 nor control plasmid displayed nuclear fragmentation, a cytological change associated with apoptosis (Figure 8B).

#### Discussion

We have identified *harakiri*, a novel regulator of apoptosis that exhibits death-inducing activity in mammalian cells. Hrk interacts with Bcl-2 and Bcl-XL, two death-repressing Bcl-2 family members that play essential roles in maintaining cell survival in embryonic and adult tissues (Veis *et al.*, 1993; Motoyama *et al.*, 1995). Hrk shares homology with Bcl-2 in the BH3 domain but, unlike most other Bcl-2 family members, it lacks conserved BH1 and BH2 regions. The BH3 region appears to represent a critical domain for interaction with Bcl-2 family members and regulation of apoptosis. For example, BH3 is required for death-promoting Bax and Bak to associate with Bcl-2 and Bcl-XL (Chittenden *et al.*, 1995a; Han *et al.*, 1996a; Simonian *et al.*, 1996; Zha *et al.*, 1996). Moreover, deletion of BH3 prevented the killing activity of Bak and expression of a 30 amino acid region of Bak containing BH3 linked to a membrane anchor sequence was sufficient for cell killing activity in transient assays (Chittenden *et al.*, 1995b). In our studies, a 16 amino acid region of Hrk encompassing BH3 was required for Hrk to interact with Bcl-2 and Bcl-XL, and its deletion eliminated or greatly reduced the killing activity of Hrk. In addition, these studies demonstrate that a region of 62 residues containing the BH1 and BH2 regions in Bcl-XL is essential for its binding to Hrk since Bcl-XS, an alternative form of



**Fig. 8.** The BH3 domain of Hrk is required for the induction of cell death. (A) 293T cells ( $1.7 \times 10^6$  per 35 mm plate) were transfected with 0.3  $\mu$ g of pRK7.GFP and 0.8  $\mu$ g of pCDNA3 (control), 0.8  $\mu$ g of pCDNA3-Flag-Hrk (Hrk) or 0.8  $\mu$ g of pCDNA3-Flag-Hrk  $\Delta$ BH3 ( $\Delta$ BH3) in the presence or absence of 6.7  $\mu$ g of pSFFV-neo, pSFFV-hBcl-2 (Bcl-2) or pSFFV-HA-hBcl-X<sub>L</sub> (Bcl-X<sub>L</sub>) in triplicate. The number of live cells expressing GFP was determined at 48 h after transfection by analysis of  $5 \times 10^3$  cells as described in Materials and methods. The results are shown as the mean of triplicate values  $\pm$  SD. (B) 293T cells transfected with 0.8  $\mu$ g of pCDNA3 (vector), pCDNA3-Flag-Hrk (Hrk) and pCDNA3-Flag-Hrk  $\Delta$ BH3 (Hrk  $\Delta$ BH3) were stained at 18 h after transfection with acridine orange and ethidium bromide to stain nuclei. Notice that nuclei of cells transfected with pCDNA3-Flag-Hrk were condensed and some of them are fragmented (arrows). In the experiment shown,  $\sim 20\%$  of the transfected cells exhibited apoptotic changes in the nuclei.

Bcl-X lacking these conserved domains, failed to associate with Hrk.

Susceptibility of a cell to apoptotic signals appears to be regulated in part by the relative levels and competing dimerizations of death-suppressing and death-promoting Bcl-2 family members (Oltvai and Korsmeyer, 1994; Sato *et al.*, 1994). How could Hrk activate cell death? Two non-exclusive models could be proposed to explain the role of Hrk in cell death. Hrk could be an effector molecule with intrinsic death-inducing activity, and death-suppressing Bcl-2 and Bcl-X<sub>L</sub> may serve as dominant inhibitors. In support of this model, overexpression of Bcl-2 or Bcl-X<sub>L</sub> inhibited the killing activity of Hrk. Alternatively, Hrk could promote cell death by inhibiting the protective activity of Bcl-2 and Bcl-X<sub>L</sub> and perhaps other functional homologs. Inactivation of Bcl-2 and Bcl-X<sub>L</sub> would require the interaction of these survival proteins with Hrk and be mediated by the formation of Bcl-2-Hrk and/or Bcl-X<sub>L</sub>-Hrk complexes. The analysis of the Hrk  $\Delta$ BH3 mutant strongly supports the latter model in that a region of 16 residues containing the BH3 domain required for Hrk to interact with Bcl-2/Bcl-X<sub>L</sub> was also necessary for Hrk killing activity. However, these studies cannot rule out that Hrk could also act as a death effector molecule as it is formally possible that the 16 amino acid deletion in Hrk directly or indirectly also affects an intrinsic death-inducing activity. Similarly, deletion mutant analysis of Bak has shown that elimination of BH3 greatly diminished the killing activity of Bak (Chittenden *et al.*, 1995b). Thus definitive evidence for the second model will require additional mutational studies and analysis of cells deficient in Hrk.

Oncogenic signals such as deregulated c-Myc or adenovirus E1A can promote or induce apoptosis in cell lines (Askew *et al.*, 1991; White *et al.*, 1991; Evan *et al.*, 1992). Thus, it is conceivable that in tumor cells, apoptotic signals are constitutively expressed, although repressed by Bcl-2 and/or Bcl-X<sub>L</sub>. Consistent with this is the observation that expression of Bcl-X<sub>S</sub>, a functional inhibitor of Bcl-2 and Bcl-X<sub>L</sub> (Boise *et al.*, 1993), activates apoptosis in a wide variety of cancer cells (Clarke *et al.*, 1995). Thus, physical inactivation of Bcl-2 and Bcl-X<sub>L</sub> by Hrk could similarly unleash endogenous death signals leading to execution of the apoptotic program. This hypothesis is consistent with recent observations in mutant mice lacking Bcl-2 and Bcl-X (Veis *et al.*, 1993; Motoyama *et al.*, 1995). Mice deficient in Bcl-X developed massive apoptosis of neural and hematopoietic tissues and died at E12–13 of embryonic development (Motoyama *et al.*, 1995), whereas newly born Bcl-2-deficient mice exhibited fulminant apoptosis of lymphoid tissues (Veis *et al.*, 1993). Thus, in addition to cancer cells, expression of Bcl-X or Bcl-2 appears necessary to counter death signals that arise during normal development. The precise mechanism of death triggered by Hrk needs to be determined, but we hypothesize that it involves the activation of interleukin 1- $\beta$  converting enzyme-like proteases, a step which appears to be downstream of Bcl-2 and Bcl-X<sub>L</sub> in the death pathway (Chinnaiyan *et al.*, 1996).

*harakiri* is the second member of a class of cell death regulatory genes that also include *bik*/*bnk*. Unlike 'classical' bcl-2 family members, the proteins that they encode lack conserved BH1, BH2 or BH4 regions but share the BH3 domain. Our studies indicate that the BH3

domain of Hrk is critical for its interaction with Bcl-2 and Bcl-X<sub>L</sub>. Although Hrk and Bik/Nbk share both a BH3 domain and a hydrophobic region at the COOH-terminus predicted to mediate attachment to intracellular membranes, these proteins are distinct and do not share additional amino acid homology. Thus, it is possible that there are subtle differences in the function of Hrk and Bik/Nbk. In this respect, it has been shown that Bcl-X<sub>S</sub> interacts with Bik/Nbk (Boyd *et al.*, 1995), but our analysis revealed that Bcl-X<sub>S</sub> fails to associate with Hrk. In addition, Hrk and Bik/Nbk differ in their pattern of expression in tissues (Boyd *et al.*, 1995), which suggests that they play distinct roles in the regulation of apoptosis *in vivo*. Future studies need to address a role for *harakiri* in the regulation of physiological cell death during tissue development and homeostasis.

## Materials and methods

### Screening for Bcl-2-interacting proteins by the yeast two-hybrid system

A HeLa cDNA library fused to the GAL4 activation domain of the pGAD-GH vector (Hannon *et al.*, 1993) was screened for proteins that interact with human Bcl-2, using the HF7c yeast reporter strain (Feilott *et al.*, 1994). Briefly, the pGAD library plasmid was transformed into HF7c yeast cells harboring the pGBT-9-bcl-2 bait plasmid by standard transfection procedures. Transformed HF7 cells were plated on medium lacking tryptophan, leucine and histidine. A total of  $3 \times 10^6$  library clones were screened for growth in selection medium and assayed for  $\beta$ -galactosidase activity. Positive clones were picked and yeast plasmid DNA from individual clones was used to transform *Escherichia coli* HB101 (leuB<sup>-</sup>) cells. Library plasmids were recovered selectively from bacterial colonies by growth in media lacking leucine. False-positive clones were eliminated by testing for interaction with empty vector pGBT-8 and irrelevant 'baits'. cDNA inserts in the plasmid were characterized by restriction enzyme mapping and nucleotide sequence analysis using an automated DNA sequencer (Applied Biosystems Model 373A).

### Plasmid construction

Plasmids expressing hBcl-X<sub>L</sub> or hBcl-X<sub>S</sub> (Boise *et al.*, 1993), mBax (Oltvai *et al.*, 1993) and hBak (Farrow *et al.*, 1995) in pGBT-9 were constructed by PCR amplification of plasmid cDNA to incorporate restriction sites, followed by ligation of the amplified DNA fragments in-frame with the GAL4 DNA-binding domain of pGBT-9. The authenticity of the GAL4 fusion plasmids was confirmed by dideoxy sequencing. The mammalian expression plasmids SFFV-Flag-hBcl-2, SFFV-Flag-hBcl-X<sub>L</sub> and SFFV-Flag-mBax have been described (Merino *et al.*, 1995; Simonian *et al.*, 1996). Three different constructs were generated to express *harakiri* sequences. In an initial construction, a Flag epitope sequence was attached to nucleotide 7 of the 5' untranslated region of the *harakiri* cDNA to generate Flag-Hrk16K by PCR using pGAD-GH-*harakiri* plasmid as a template (see Figure 3A). The Flag sequence in Hrk16K was in-frame with the amino-terminus of Hrk, resulting in a fusion protein with a predicted size of 16 kDa. In a second construction, a Flag-Hrk insert was constructed by introducing a Flag epitope tag at the amino-terminus of Hrk by PCR. A deletion of amino acids 34–49 that includes the BH3 domain of Hrk was generated by a two-step PCR mutagenesis method as described (Simonian *et al.*, 1996). A Flag epitope tag was attached to the amino-terminus of mutated Hrk protein to generate Flag-Hrk  $\Delta$ BH3. A Flag epitope tag was attached to the amino-terminus of a control cDNA clone BP25 (N.Inohara, L.Ding and G.Núñez, unpublished data) by PCR. The Flag-tagged inserts were ligated into the *Eco*RI cloning site of pSFFV-neo or the *Bam*HI and *Xho*I sites of pCDNA3 (Invitrogen, San Diego, CA). Orientation of the inserts was determined by restriction mapping. The authenticity of all Flag-tagged constructs was confirmed by dideoxy sequencing. An expression plasmid that produces a Flag-tagged GATA-1 protein was obtained from Dr Vishva Dixit (Department of Pathology, University of Michigan).

### Screening of human cDNA library by hybridization with labeled probe

A 9-week human embryo  $\lambda$ gt11 cDNA library (Swaroop and Xu, 1993) was screened by hybridization with a <sup>32</sup>P-labeled cDNA containing the entire coding region of *harakiri*. Approximately  $1 \times 10^6$  cDNA clones were screened by standard procedures, and positive phage clones were purified by sequential plating and hybridization. DNA inserts were characterized by PCR amplification of phage DNA from purified plaques, restriction enzyme mapping of phage DNA inserts and dideoxy sequencing.

### Transfection, immunoprecipitation and Western blot analysis

Human embryonic kidney 293T and 293 cells were obtained from Dr Vishva Dixit (Department of Pathology, University of Michigan). Culture dishes containing cells were transfected with the indicated amount (see figure legends) of plasmid DNA by the calcium phosphate method. The expression of Flag-Bcl-X<sub>L</sub>, Flag-Bcl-2, Flag-Hrk, Flag-Hrk  $\Delta$ BH3 and Bax was determined in total lysates by Western blot analysis as previously described (Merino *et al.*, 1995; Simonian *et al.*, 1996). For immunoprecipitations,  $1 \times 10^7$  cells were lysed in NP-40 isotonic lysis buffer (Oltvai *et al.*, 1993) at 38 h after transfection and the lysates were rotated with 10  $\mu$ g/ml of anti-Flag M2 monoclonal antibody (Scientific Imaging Systems, Rochester, NY), hamster anti-Bcl-2 monoclonal antibody (Hockenbery *et al.*, 1991), rabbit anti-Bcl-X (Boise *et al.*, 1995) or control Ig overnight at 4°C. Then 5% (v/v) of protein A-Sepharose 4B (Zymed Laboratories Inc., San Francisco, CA) was added for an additional hour of incubation by rotation. Immune complexes were centrifuged and washed with excess cold NP-40 isotonic lysis buffer at least four times, separated on a 15% SDS-polyacrylamide gel and immunoblotted with anti-Bcl-2, anti-Flag, rabbit anti-Bcl-X or control Ig.

### Northern blot analysis

A 716 bp *harakiri* cDNA was radiolabeled by the random priming method using a commercial kit (Boehringer Mannheim, Indianapolis, IN) and applied for analysis of human multiple tissue blots (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions.

### Laser scanning confocal microscopy and fluorescence staining of nuclear DNA

293 cells were transfected with pCDNA3-Flag-Hrk, pCDNA3-Flag-GATA-1, or empty vector as described above. Twenty four hours after transfection, cells were incubated with anti-Flag monoclonal antibody or control mouse Ig for 1 h at 23°C and the labeling visualized with fluorescein-conjugated goat anti-mouse IgG. After washing, the cells were mounted in Slowfade (Molecular Probes, Eugene, OR) and examined using a BioRad MRC 600 scanning confocal microscope equipped with an argon-xenon laser (González-García *et al.*, 1994). Staining of nuclei with acridine orange and ethidium bromide was performed as described previously (Duke and Cohen, 1994).

### Cell death assays

293T, 293 and HeLa cells were co-transfected by a calcium phosphate method with pRK7.GFP, a reporter plasmid expressing GFP (a gift of Roger Y.Tsien), in combination with an expression plasmid encoding Hrk, Bcl-2, Bcl-X<sub>L</sub> or control plasmid. The number of plasmids transfected is indicated in the figure legends. Killing activity was determined at 48 h after transfection and based on the analysis of  $5 \times 10^3$  cells by FACSscan flow cytometry (Becton Dickinson, Mountain View, CA). In the assay, the cell killing activity was manifested by a reduction in the number of cells that express GFP relative to that obtained by transfection with the control expression plasmid.

### Accession number

The accession number for the nucleotide sequence of *harakiri* cDNA reported in this paper is U76376.

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